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Preparation of cyclodextrin nanoparticles and evaluation of its effect on the capacitation of bovine spermatozoa used in the *in vitro* fertilization

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Abstract

This study was conducted to produce nanosized cyclodextrin (NCD) and assess its effect on bovine spermatozoa during In vitro fertilization (IVF) to optimize the capacitation media for successful IVF. Therefore, four cyclodextrin formulations were prepared and characterized. Data analysis revealed the best formula (F2) showed a smallest particle size (15 nm), zeta potential (-37 mv), and higher yield percentages (95%) was selected for spem capacitation. Motile spermatozoa were separated from frozen-thawed semen by a swim-up procedure and capacitated in IVF-TALP medium with different formulae of NCD or CD or without treatments (control) and incubated for 3hours(hr) at 38 C and evaluated every one (hr) interval. Data analysis revealed that the formulation of cyclodextrin nanoparticles (F2) after (2hr) incubation in the media gave best effect on sperm capacitation and acrosme reaction (AR) and effect of sperm treated with NCD on fertilization rate was evaluated. The results showed that the proportion of Oocytes fertilized was increased significantly in F2 (60%) than in the control (35%), and cyclodextrin group (50%) groups ($p<0.05$). It could be inferred from this investigation that cyclodextrin nanoparticles can be used for biomedical interventions in bovine spermatozoa. NCD improve sperm motility, viability, and (AR), also fertilization rate of sperm treated with NCD increase. So NCD gave positive effect on sperm functions during IVF.

Keywords: Capacitaion; cyclodextrin; nanoparticles; In Vitro Fertilization; spermatozoa.

Introduction

Nanoparticle is defined as a particle with dimensions between 1 and 100 nano-meters, [1]. Nanosized particles differ from larger samples of the same material in their chemical and physical properties such as ultra small size, large surface area to mass ratio and high reactivity. These advantages in properties improve the limitation of use of traditional substancces [2]. Also the very small size of nanoparticles facilitates their penetration through very small capillaries into the human tissues and cell.

Sperm capacitation is a biochemical process which is associated with membrane changes like cholesterol efflux, alteration of plasma membrane and further increase the membrane fluidity and also has great effect on hyperactivation (HA) and acrosome reaction (AR) of spermatozoa [3]. Sperm must undergo capacitation to fertilize the oocyte

There are many inducers for acrosome reaction after complete capacitation in mammalian sperm. Pharmacological inducers include heparin [4] and calcium ionophore A23187 [5] and Physiological inducers like zona pellucid have been used in studies to activate the acrosome reaction.

In this study we add CN nanoparticles to capacitation media to evaluate the biological effect of nanoparticles on bovine sperm during IVF Cyclodextrins functions as carrier molecules and dissolves lipophiles in their hydrophobic core [6]. So CD as nanoparticles stimulate efflux of cholesterol from sperm and improve capacitation of sperm consequently improve acrosome reaction, and fertilization rate. Evaluation of effect of nanoparticles in vitro make us can predict their effect in vivo [7].

Materials and Methods

Materials

All reagents, Chemicals and Media were from Sigma-Aldrich unless differently specified.

Methods

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Experiment I: Preparation of Cyclodextrin Nanoparticles (CDN).

Cyclodextrin nanoparticles (CDN) were spontaneously obtained via ionotropic gelation method [8] with modification. Four formulae were prepared according to CD: Alginate ratio as described below in Table 1.The best formula with smallest particle size and highest yiel percentage was selected for spem capcitation.

Table 1. Formulations of cyclodextrin nanoparticles.

Formula(NO)	Cyclodextrin : Alginate ratio
F1	1 CD : 8 AG
F2	2 CD : 8 AG
F3	4 CD : 8 AG
F4	8 CD : 8 AG

The morphology of the CD nanoparticles were characterized by Transmission Electron Microscope (TEM) JEM-2100 (JEOL- td, Tokyo, Japan), the z-average particle sizes were determined using the PCS4700 system (Malvern Instruments, Malvern, UK), Zeta potential of the nanosuspension samples was determined using the Zeta Sizer 2000 (Malvern Instruments), The CD nanoparticles were separated by centrifugation and then freeze- dried using Freeze – Dryer, FDu-7003, (Kyeonggi-do, Korea) to yield the product.
The nanoparticles product yield was calculated as follows:

$$\text{Yield of nanoparticles} = \frac{\text{Weight of Nanoparticles}}{\text{Weight of initial CD/ALG/Ca2+}} \times 100$$

Experiment II: Effect of nanosized cyclodextrin on sperm capacitation

Separation of motile spermatozoa

One 0.25 ml straw of frozen semen was thawed in a water bath at 37◦C for 10 seconds(s) were separated using a modified swim-up method. Thawed sperm were added to 1ml of SP-TALP. Sperm were carefully deposited under 1 ml equilibrated (38.8 C in 5% CO2) modified Tyrode's medium for sperm isolation (SP-TALP) in the bottom of the tube and incubated at 38.8 C in 5% CO2 for one hour. After incubation, the bottom part of the medium were removedand the upper part with motile sperm was centrifuged twice at 200 G (1000 rev/min) for 10. The pellet was diluted with the fertilizing IVF-TALP medium to give the final concentration of 20 10^6 sperm per ml of medium (sperm suspension).

Treatment of bovine spermatozoa with nanosized cyclodextrin

Sperm suspension was divided into 6 equal sizes in 6 test tubes (4 tubes for 4 different types of cyclodextrin nanoparticles formulations and one tube for cyclodextrin and one without treatment, control). After that, all test tubes were incubated at 39ºC for (3hr).

Assessment of Sperm Capacitation

Assessment of the hyperactivation motility

Assement of Hyper-activation Motility (HAM) were performed according to the method of Fujinoki et ., al [9] with some modifications. It Motile spermatozoa suspended in the IVF-TALP medium were recorded on TV via a camera attached to a microscope (Olympus, Japan) with hot stage at 1,2 and 3 hr incubation period. Each observation was performed at 37ºC, recorded for 2 min, and analyzed by manually counting the numbers of total spermatozoa, motile spermatozoa and hyperactivated spermatozoa in 10 different fields. Experiments were performed 10 times using 10 semen samples.

Assessment of Sperm Viability

Assessment of bovine spermatozoa viability by means of Trypan Blue exclusion method. A 100µL aliquot of sperm suspension (10^8cells/ml) was mixed with an equal volume of 2% trypan blue for 15 min at 39ºC then live cells were counted by hematocytometer [5].

Assessment of acrosome reaction (AR)

The Coomassie Blue G staining is used to evaluate acrosome reaction[10]

Transmission Electron Microscope Analysis (Localization of the Cyclodextrin).

Localization of NCD in bovine spermatozoa was viewed by TEM.after (2hr) of treatment with NCD. Untreated sperm cells served as a control.

Experiment III: The fertilizing ability of spermatozoa

According to experiment 2 results, the best formula which has the shortest times of incubation with the highest rate of sperm capacitation was chosen for fertilization procedure.

Oocyte collection and culture

The collected ovaries from slaughterhouse, kept in physiological saline (32–35°C), and transported to the laboratory within 45 min an prepared for maturation (24h) according to [11] .

In vitro fertilization

For fertilization in-vitro groups of matured Oocytes in micro droplets of fertilization medium were inseminated with(1-1.5 million) sperm /ml of capacitated sperm to (IVF-TALP) and then cultured for (24hr) at 39 C in 5% CO2 .after 24 hr examined for fertilization. Matured oocytes were divided randomly into 3 different groups. The first group the cumulus-enclosed was kept after in vitro maturation (IVM) and fertilized in IVF-TALP medium (group 1: Control). The other groups were fertilized in IVF-TALP medium supplemented with 100 ul /ml CD (group 2: IVF-TALP+ CD), IVF-TALP medium supplemented with 100 ul /ml cyclodextrin Nanoparticles (group 3: IVF-TALP+NCD, F2), respectively. The experiment was replicated ten times.

Assessment of fertilization

Fertilization criteria were those described previously [12] and presence of second polar bodies, 2 pronuclei or cleavage to at least the 2-cell stage indicated that fertilization take place

Statistical analysis

Data are represented as the mean ± S.D. One-way ANOVA test and post hoc comparisons using Tukey's test (SPSS18 software; SPSS, Inc., Chicago, IL, USA) were used to analyze the significant differences among the groups. Differences were considered significant at $p<0.05$

Results and Discussion

Characterizations of different formulations of cyclodextrin nanoparticles

Detailed characterizations of different formulations of cyclodextrin are briefly shown in Table 2. Cyclodextrin nanoparticles were almost spherical in shape and homogenous as seen under TEM.
Comparing all cyclodextrin formulations indicated that the NCD (F2) are better than other formulations in terms of yield percentages in addition, the average particle size and zeta potential decreased in NCD (F2) ($P > 0.05$).

Table 2. Characterization of different formulations of cyclodextrin.

Formula (NO)	Structure of Formula	particle size(nm)	zeta potential (mv)	Yield (%)
F1	1 CD : 8 AG	25	-50	90%[a]
F2	2 CD : 8 AG	15	-47	95%[b]
F3	4 CD : 8 AG	30	-42	85%[c]
F4	8 CD : 8 AG	50	-27	55%[d]

Data were expressed as Means ± SE; different alphabetical superscripts in the same rows (a, b, c, d) are significant at least at P < 0.05.

Morphology of the optimal formulation (F2) is shown in Figure 1

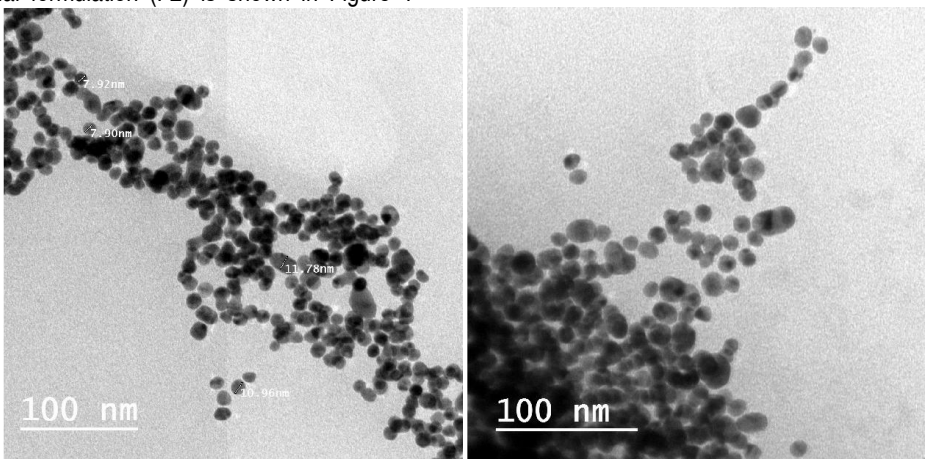


Figure.1: TEM micrograph CD nanoparticles (F2).

In vitro sperm capacitation

Treatment effect: HAM, Viability and AR were affected by treatment.

A greater HAM and AR spermatozoa was observed for nanosized cyclodextrin ($P < 0.05$) (Table 3).

Time effect: The best time for HAM and AR was (2 hr) and for viability was (1hr) as compared with other incubation times (Tables 4,5,6).

Table 3: Effect of nanosized cyclodextrin on Hyper-activitated Motility, Viability and Acrosme Reaction of bull spermatozoa during sperm capacitation after 3 h incubation time.

Treatments	Time	Con.	CD	NCD			
				F1	F2	F3	F4
Hyper-motility (%)	3 h	20^{aC}	30^{bC}	35^{bC}	50^{cC}	15^{dC}	10^{dC}
Viability (%)	3 h	10^{aC}	30^{bC}	40^{bC}	55^{cC}	15^{dC}	10^{dC}
Acrosme Reaction (%)	3 h	25^{aC}	35^{bC}	40^{bC}	55^{cC}	15^{dC}	10^{dC}

^{a,b,c,d} Mean values within a row with different superscripts differ significantly ($P<0.05$).
^{A,B,C} Means values within a column with different superscripts differ significantly ($P<0.05$).
Con. = Control contains sperm cells and devoid of nanoparticles.
CD = Cyclodextrin ,NCD = Nanosized Cyclodextrin

Table 4. Effect of nanosized cyclodextrin and incubation time (hours) on hyperactivity of bovine spermatozoa.

Treatments	Time	Con.	CD	NCD			
				F1	F2	F3	F4
Hyperactivated Motility (%)	1 h	25^{aA}	40^{bA}	45^{bA}	65^{cA}	30^{dA}	20^{Da}
	2 h	40^{aB}	50^{bB}	55^{bB}	75^{cB}	20^{dB}	15^{dB}
	3 h	20^{aC}	30^{bC}	35^{bC}	50^{cC}	15^{dC}	10^{dC}

^{a,b,c,d} Mean values within a row with different superscripts differ significantly ($P<0.05$).^{A,B,C} Means values within a column with different superscripts differ significantly ($P<0.05$).Con. = Control contains sperm cells and devoid of nanoparticles
CD = Cyclodextrin .NCD = Nanosized

The changes in the motility rate and pattern of movement of spermatozoa under the influence of NCD were clarified by analyzing the change in movement pattern after incubation for 1 and 3 hr in different formulations of cyclodextrin nanoparticles.

As shown in Table 4, the percentages of HAM were severely depressed at higher concentrations of cyclodextrin nanoparticles formula NCD (F4) and longer time of incubations, whereas lower concentrations in other formulae NCD (F2) enhanced motility and the best motility was maintained at 2hr of incubation.

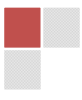
Effect of NCD on Sperm Viability

The results obtained for viability which was evaluated by Trypan Blue stain presented in (Figure.2) a significant difference ($P<0.05$) in the percentage of live sperm in the treatment that increased in concentration of cyclodextrin nanoparticles at 1, 2 and 3h incubation (Table 5).

Table 5. Effect of different formulations of nanosized cyclodextrin and incubation time (hours) on viability of bovine spermatozoa.

Treatments	Time	Con.	CD	NCD			
				F1	F2	F3	F4
Viability (%)	1 h	35^{aA}	55^{bA}	60^{bA}	75^{cA}	40^{dA}	30^{dA}
	2 h	25^{aA}	45^{bB}	50^{bB}	70^{cB}	25^{dB}	20^{dB}
	3 h	10^{aC}	30^{bC}	40^{bC}	55^{cC}	15^{dC}	10^{dC}

^{a,b,c,d} Mean values within a row with different superscripts differ significantly ($P<0.05$).
^{A,B,C} Means values within a column with different superscripts differ significantly ($P<0.05$).
Con. = Control contains sperm cells and devoid of nanoparticles
CD = Cyclodextrin, CDN = Cyclodextrin Nanoparticles



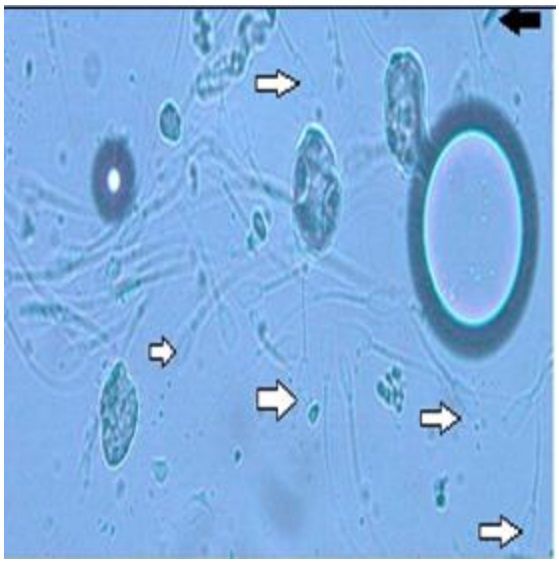


Figure.2: Frozen-thawed bovine spermatozoa stained with Trypan Blue after capacitation. Live sperm (open arrowheads) lack the head of sperm staining and the sperm is more lightly stained while Dead sperm (filled arrowheads) display a prominent dark blue head sperm.

Effect of NCD on Sperm Acrosome reaction

AR which was evaluated by Coomassie Blue G stain (Figure.3) was significantly increased in NCD (F2) at 2h incubation ($P<0.05$) as compared to others formulae and control (Table 6).

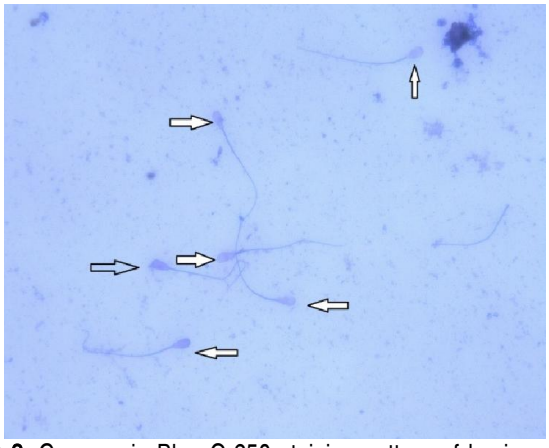


Figure.3: Coomassie Blue G-250 staining pattern of bovine sperm (X400).

in Figure Intact sperm (filled arrowheads) display a prominent dark blue apical ridge along the anterior border of the head while AR sperm (open arrowheads) lack the apical ridge staining and the acrosomal region of the head is more lightly stained than the post-acrosomal head region.

Table 6. Effect of nanosized cyclodextrin and incubation time (hours) on acrosome reaction of bovine spermatozoa

Treatments	Time	Con.	CD	NCD			
				F1	F2	F3	F4
Acrosome Reaction (%)	1 h	30^{aA}	50^{bA}	50^{bA}	70^{cA}	25^{dA}	20^{dA}
	2 h	45^{aA}	55^{bB}	60^{bB}	80^{cB}	20^{dB}	15^{dB}
	3 h	25^{aC}	35^{bC}	40^{bC}	55^{cC}	15^{dC}	10^{dC}

^{a,b,c,d} Mean values within a row with different superscripts differ significantly ($P<0.05$).
^{A,B,C} Means values within a column with different superscripts differ significantly ($P<0.05$).
Con. = Control contains sperm cells and devoid of nanoparticles
CD = Cyclodextrin, NCD = Nanosized Cyclodextrin

Table 7. Fertilization rate according to the results of hyperactivated motility (HAM) and acrosome reaction (AR) of nanozsized cyclodextrin.

Treatments	No. of Inseminated Oocytes	Fertilized Oocytes	Fertilization Rate (%)
Control	100	35	35^{a}
Cyclodextrin	100	50	50^{b}
Nanosized Cyclodextrin	100	60	60^{c}

^{a,b,c} Means values within a column with different superscripts differ significantly ($P<0.05$). n=10 for each treatment.

Transmission Electron Microscopic Analysis of Bovine Spermatozoa Treated with nanosized cyclodextrin.

Bovine spermatozoa incubated with and without nanosized cyclodextrin at (2 hr) were viewed under TEM. NCD were characterized by spherical in shape. TEM analysis allowed for

highlighting similar particles into bovine spermatozoa and outside (in the surrounding medium) (Figure.4). In the control samples these particles were not seen.

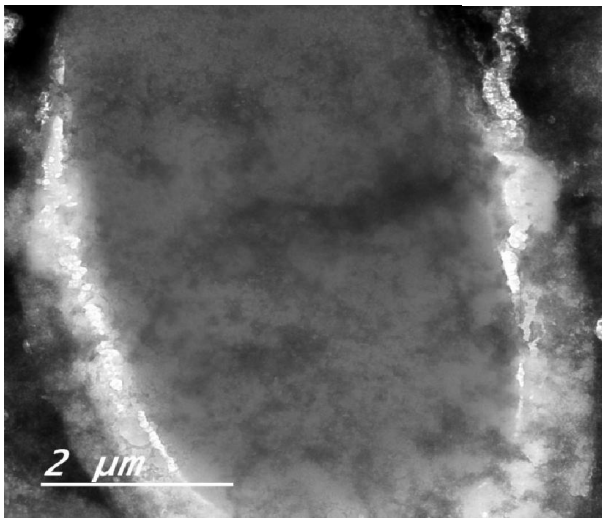


Figure.4: TEM-micrographs of bovine spermatozoa after co-incubation with cyclodextrine nanoparticles (F2) for 2 h at 37 C.

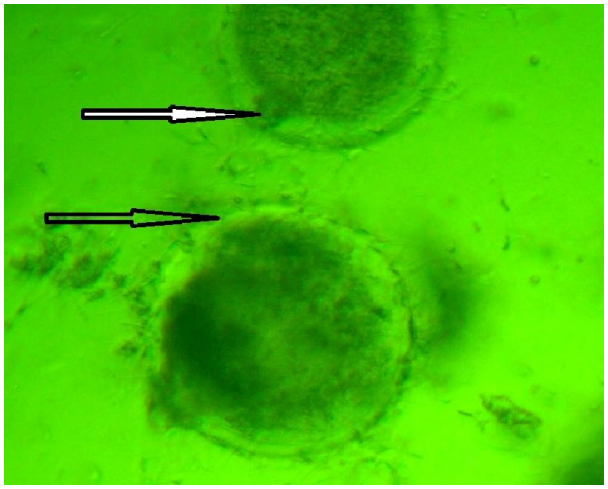


Figure.5: A representative fertilized 117ocytes after 22 h fertilization. Note the second polar body (Filled Arrow) and 2 cell fertilized oocytes (open arrow).

Experiment III: in vitro fertilization

A summary of the results of in vitro fertilization is shown in (Table 7).

The fertilization rate was highest at NCD (60%) than for control and CD (35% and 50% respectively). (Figure.5)

This is the first report of the effect of NCD on the in vitro capacitation of bovine spermatozoa. The morphology and size of the cyclodextrin nanoparticle prepared by ionic gelation was characterized by transmission electron microscopy (TEM). Comparing four cyclodextrin formulations, Data analysis revealed that all formulae of cyclodextrin showed an average particle size below 100 nm and this agreement with the standard dimensions reported by many organizations such as ISO, ASTM, NIOSH, SCCP, BSI and BAuA [1] . But the best ratio of the highest yield and smallest particle size was 2:8 CD/ALG (CDN_2). CD used in many applications [13]

As shown in Table 3, the percentages of HAM and AR were highly significant in nanosized cyclodetrine (F2) compared to the control and other formuale treatments ($P<0.05$).

The viability results presented a significant difference ($P<0.05$) in the percentage of live sperm after (3hr) of incubation in the treatment that received NCD.

In order to induce the acrosome reaction, culture media for sperm capacitation are supplemented with different agents, in cattle preferentially with progesterone, which stimulates capacitation of bovine spermatozoa [14, 15, 16]. Authors who have described the behavior of spermatozoa during capacitation and acrosome reaction onset reported that the first changes in acrosome morphology appear after 2 hr [17]. We observed initial morphological changes in acrosomes earlier, We assume that this difference is due to the use of a nanosized cyclodextrin in our study instead of cyclodextrin as nanoparticles differ from normal cyclodextrin in their properties So the nanosized cyclodextrin are small enough to penetrate through sperm plasma membrane. Because cyclodextrin nanoparticles can pass through sperm plasma membrane, they have great effect on of the sperm functions. It was found that successful in vitro fertilization in mice by addition of methyl-b-cyclodextrin to capacitation media methyl which induce efflux of cholesterol and induce capacitation of mice sperm as described in [18] In our study nanoparticles improve the effect of cyclodextrin on sperm functions more than normal cyclodextrin

Our results showed that with prolongation of time for 3 hr, cyclodextrin treated sperm lost its hyperactivated pattern and viability as described in [19, 20].

Conclusions

Based on the finding of the present study, it can be concluded that incorporation of CD into nanoparticulate improve its properties by increasing the surface area also ultra small particle size facilitate the penetration of CN to the sperm which led to improve sperm functions. So incubation of bovine spermatozoa with NCD (F2) the selected formula which showed smallest particle size and highest yield percentage for 2hr revealed increased sperm hyper-motility and acrosome reaction as well as it produced the highest percentage of fertilized oocytes as compared to cyclodextrin or control. So this study is considered a promise to treat male infertility due to bad quality of sperm during IVF, and further studies to different types of mammalian sperm are needed.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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